INHIBITION OF MITOCHONDRIAL CARBAMYL PHOSPHATE SYNTHETASE IN RAT AND HUMAN LIVER BY ACYL CoA ESTERS: A POSSIBLE MECHANISM FOR HYPERAMMONEMIA IN THE INHERITED ORGANIC ACIDEMIAS

Jeffrey Alan Gruskay

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INHIBITION OF MITOCHONDRIAL CARBAMYL PHOSPHATE SYNTHETASE IN RAT AND HUMAN LIVER BY ACYL CoA ESTERS: A POSSIBLE MECHANISM FOR HYPERAMMONEMIA IN THE INHERITED ORGANIC ACIDEMIAS

BY

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For my parents

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CONTENTS

ABBREVIATIONS

ATP, Adenosine triphosphate

Cbl, Cobalamin (vitamin B_{12})

CoA, Coenzyme A

CPS I, mitochondrial carbamyl phosphate synthetase

CPS II, cytosolic carbamyl phosphate synthetase

GLC, gas-liquid chromatography

MW, molecular weight

NAG, N-acetylglutamate

NPG, N-propionylglutamate

OTC, ornithine transcarbamylase

PCC, propionyl CoA carboxylase

Abstract

Hyperammonemia is a frequent complication of inherited deficiencies of β-ketothiolase, propionyl CoA carboxylase, and methylmalonyl CoA mutase. To probe the biochemical basis for such ammonia accumulation, I have determined whether a variety of organic acids (and their CoA esters) inhibit urea cycle enzyme activities in vitro in several preparations of rat liver (crude homogenates, isolated mitochondria, and pure enzyme) and in crude homogenates of human liver. Neither tiglate, propionate, nor methylmalonate at concentrations as high as 10 mM inhibited carbamyl phosphate synthetase I (CPS I) or ornithine transcarbamylase (OTC) activities in rat liver homogenates. Whereas the CoA esters of these organic acids also failed to impair OTC and arginase activities in this tissue preparation, they produced a significant, reproducible, concentration- and time-dependent inhibition of CPS I activity. This effect was not overcome by addition of the CPS I activator, N-acetylglutamate (NAG), hence it was not secondary to the previously reported inhibition of NAG synthetase by several organic acids. Michaelis-Menten analysis showed that propionyl CoA, the most potent inhibitor, decreased the V_{max} but did not alter the K_m's for each of the CPS I substrates (ATP, ammonium, and bicarbonate). A concentration-dependent inhibition of CPS I by propionyl CoA was also demonstrated in isolated mitochondria and pure enzyme from rat liver as well as homogenates from human liver. The addition of dithiothreitol to the pure enzyme prior to assay resulted in a decrease in the degree of inhibition seen with propionyl CoA, implying that sulfhydryl groups are involved in the inhibitory effect. These data suggest that the

hyperammonemia seen in infants with these disorders may result, in part, from direct inhibition of CPS I by these acyl CoA esters.

Part I: The Organic Acidemias and Hyperammonemia: A Review

Inborn errors of amino acid metabolism include such diverse disorders as phenylketonuria and tyrosinemia, which result from defects of aromatic amino acid metabolism; cystathionemia and homocystinuria, which result from defects of sulfur-containing amino acid metabolism; and maple syrup urine disease and isovaleric acidemia, which result from defects of branched-chain amino acid metabolism. Most of these aminoacidopathies are associated with high morbidity and mortality which are related to the accumulation of substrates proximal to the defect of the single enzymatic step which characterizes each disease (1). Early identification of these disorders may allow amelioration of the morbidity and mortality by decreasing the amount of the offending amino acids in the diet.

Disorders of branched-chain amino acid oxidation in which amino acids do not accumulate have been called the "organic acidemias". Since the initial steps of this pathway are first a transamination to the corresponding keto acid and then an irreversible decarboxylation to the corresponding organic acid, any block distal to this decarboxylation step will result in the accumulation of these organic acids, but not of the parent amino or keto acids (2).

The discovery of the inherited "organic acidemias" was made possible by the development of new techniques for identification and characterization of these organic acids, namely gas-liquid chromatography (GLC) and mass spectometry in conjunction with GLC (3). In 1966, Tanaka and colleagues (4) first used these techniques to identify isovaleric acid

in the blood and urine of a patient with the "distinct odor of sweaty feet" and a constellation of symptoms now considered to be characteristic for several of these organic acidemias. This clinical presentation includes episodes of ketoacidosis with vomiting and lethargy, and coma or other neurological manifestations (3). The particular neurological abnormalities observed include: a Werdnig-Hoffman-like syndrome with infantile paralysis in a patient with β-methylcrotonyl CoA carboxylase deficiency (5); ataxia and choreoathetosis in a patient with pyruvate carboxylase deficiency (6); and a familial dysautonomia-like picture with an abnormal histamine stimulation test in a patient with propionyl CoA carboxylase (PCC) deficiency (7). The cause of these neurological problems is unknown but they may reflect either a general neurological dysfunction secondary to severe metabolic acidosis or a specific dysfunction as in the case of PCC deficiency where increased amounts of propionyl CoA may substitute for acetyl CoA in fatty acid biosynthesis leading to the production of an abnormal myelin.

Among the many recognized organic acidemias, three, α-methylacetoacetic acidemia, propionic acidemia, and methylmalonic acidemia, share many clinical and biochemical features. First, each results from a deficiency of one of three mitochondrial enzymes, β-ketothiolase, propionyl CoA carboxylase, and methylmalonyl CoA mutase, respectively, which catalyze successive steps in the major pathway of propionate metabolism. Second, each of these disorders may present with a similar clinical picture-- severe acidosis with lethargy, vomiting and failure to thrive, mental and physical developmental retardation, hematologic abnormalities including neutropenia, anemia, and thrombocytopenia, and osteoporosis. Third, each has been associated with hyperglycinemia.

Prior to the elucidation of the precise biochemical defect in these disorders, children with deficiencies of each of these enzymes were said to have the "ketotic hyperglcinemia syndrome". In addition to hyperglycinemia, these disorders are characterized by other biochemical abnormalities, including hypoglycemia, long-chain ketonemia and ketonuria, and, as will be discussed further, hyperammonemia. The cause of these biochemical derangements is unknown, but they are almost certainly seondary to the primary deficiency in organic acid degradation (8).

To understand the relationship between these primary defects and the secondary biochemical abnormalities, it is necessary to examine the major biochemical pathway of propionate metabolism. (See Figure 1) Isoleucine is transaminated and decarboxylated to the organic acid, α-keto-β-methylvaleric acid which undergoes further metabolism including addition to a Coenzyme A carrier and dehydrogenation to yield α-methylacetoacetyl CoA. The latter intermediate is split by β-ketothiolase to yield propionyl and acetyl CoA's. Other compounds including valine, threonine, methionine, odd chain fatty acids, and cholesterol side chains are also metabolized to propionyl CoA. Previously, it was thought that valine entered the Kreb's Cycle through methylmalonyl CoA directly, but recent work has provided evidence that propionyl CoA is an obligate intermediate in this pathway (9). Propionyl CoA is carboxylated to D-methylmalonyl CoA by propionyl CoA carboxylase, a biotin requiring enzyme (10). Because only the L-isomer of methylmalonyl CoA is a substrate for methylmalonyl CoA mutase, D-methylmalonyl CoA undergoes racemization to the L-isomer in a reaction catalyzed by methylmalonyl CoA racemase. Finally, L-methylmalonyl CoA undergoes isomerization to succinyl CoA in a reaction catalyzed by methylmalonyl CoA mutase, a

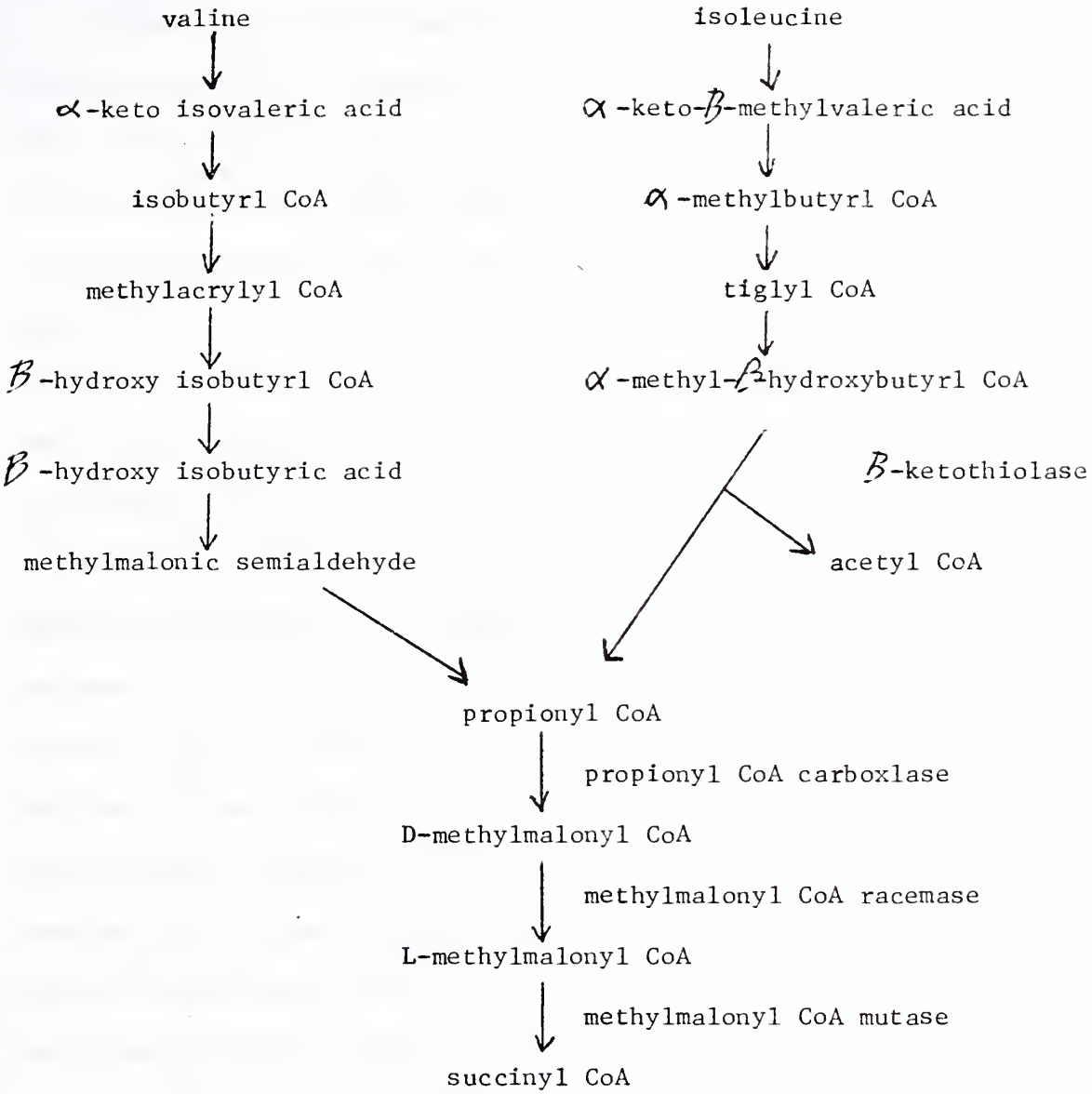

Figure 1. Metabolism of the branched-chain amino acids valine and isoleucine

vitamin B_{12}-requring enzyme (11).

Vitamin B_{12}, or more appropriately cobalamin (cbl), metabolism has been extensively studied and this work is important in understanding the different abnormalities that can result in methylmalonic acidemia. Cbl is a cobalt containing vitamin with six cobalt-associated ligands; it occurs in a natural form, hydroxocobalamin (OH-Cbl), and a synthetic form, cyanocobalamin (CN-Cbl). These forms of cbl are not active as coenzymes but must be converted by the body to their active form by modification of the sixth ligand. Cbl absorption in the terminal ileum is dependent on the presence of a protein produced by the gastric parietal cells, intrinsic factor. The intrinsic factor- cbl complex binds to the mucosa of the terminal ileum, finally resulting in the release of free cbl into the blood. There, the cbl is bound to a serum protein carrier, transcobalamin II, which transports it to the cells of the body for metabolism and use. (See Figure 2) Once inside the cells, the cobalamin vitamin is transported into the mitochondria where it undergoes two enzyme-catalyzed reductions and, finally, adenosylation to yield 5'-deoxyadenosylcbl (Ado-Cbl), the active cofactor for methylmalonyl CoA mutase activity. OH-Cbl can also be converted, by a pathway which has not yet been elucidated, to methylcobalamin (Me-Cbl), the cofactor for N^5-methyltetrahydrofolate: homocysteine methyltransferase, a key cytosolic enzyme in the pathway of sulfur-containing amino acid degradation and folate interconversion.

Figure 2. Intracellular metabolism of OH-Cbl to active cofactors. (see following page) OH-Cbl is transported to the cell by transcobalamin II. Once inside the cell, OH-Cbl enters the mitochondrion where it is metabolized to Ado-Cbl, the active cofactor for methylmalonyl CoA mutase. Alternatively, OH-Cbl may remain in the cytosol where it is metabolized to Me-Cbl (by a pathway which has not yet been elucidated).

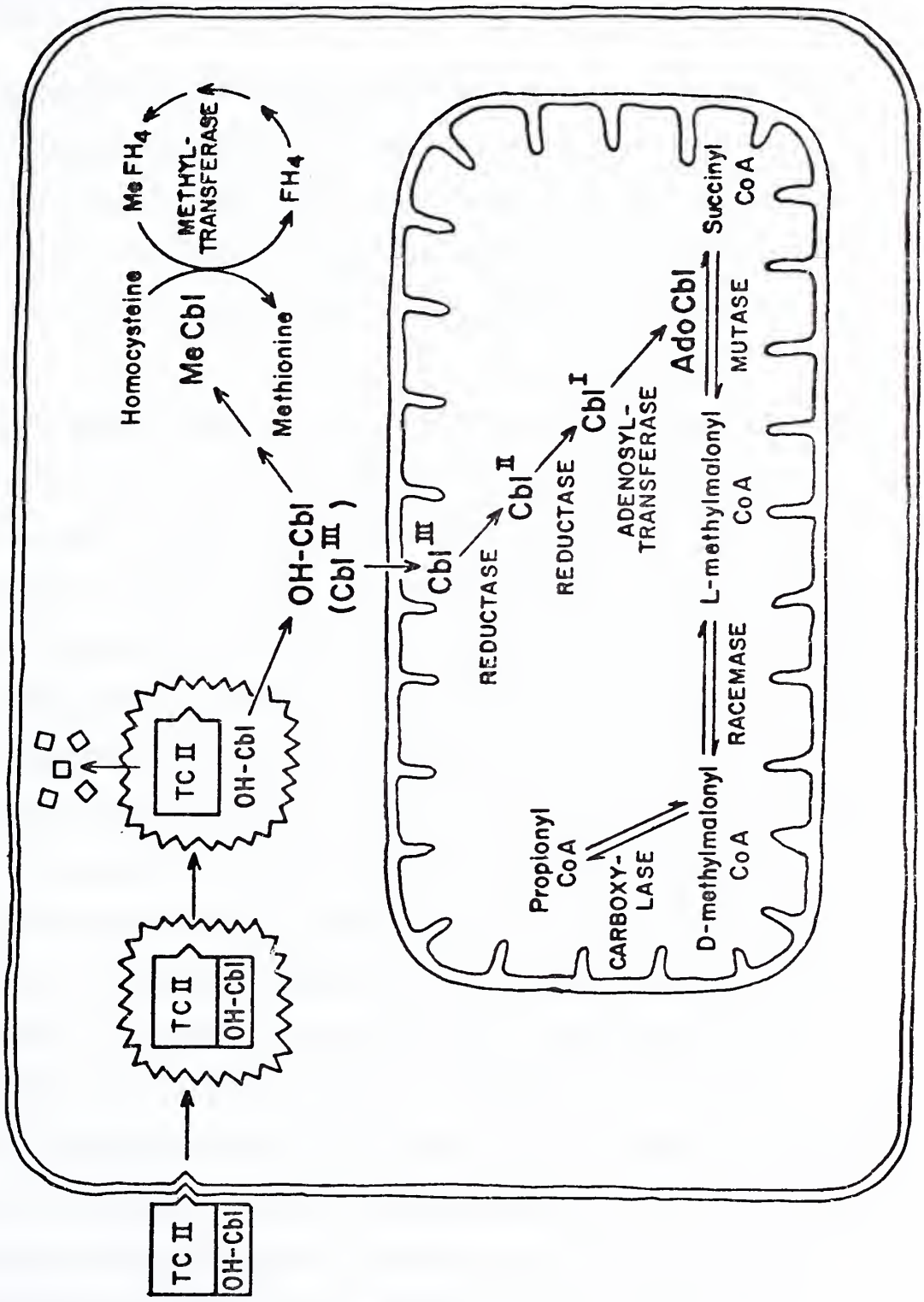

Figure 2. Intracellular metabolism of OH-Cbl to active cofactors. (see figure legend on preceeding page)

Methylmalonic Acidemia

As mentioned above, the organic acidemias are characterized by several secondary biochemical derangements, such as hyperammonemia, hypoglycemia, and hyperglycinemia. Proof that these derangements are indeed secondary to defects in organic metabolism first required the demonstration of the primary defects in the pathway of propionate metabolism.

It has long been known that cbl is required for methylmalonyl CoA mutase activity (11); thus, the increased amounts of methylmalonic acid in the urine of patients with pernicious anemia (12), and with cbl deficiency for other reasons (13) were expected. In these reports, parenteral administration of physiologic amounts of cbl led to a dramatic fall in methylmalonate excretion. However, in 1967, two groups (14,15) described patients with increased methylmalonate excretion who had normal serum cbl concentrations. Stokke and colleagues (14) described a child who presented on the second day of life with irritability, a weak cry, decreased activity, hypotonia and irregular respirations who was found on laboratory examination to have severe metabolic acidosis with ketonuria, and transient thrombocytopenia. Methylmalonic acid was identified in this patient's urine by GLC and mass spectometry. The patient was given a trial of cbl without clinical response or decreased methylmalonate excretion. Further laboratory work in this child revealed that C^{14}-valine loading resulted in excretion of C^{14}-methylmalonate and this led the investigators to postulate that the accumulation of methylmalonate was secondary to a defect in protein catabolism. The

patient was started on a diet low in methylmalonate precursors (isoleucine, valine, threonine, and methionine) and this resulted in improvement of the clinical condition and reduction in the tendency toward acidosis. With this diet, the excretion of methylmalonic acid fell from 800-1000 mg/24 hours to 100-200 mg/24 hours (normal less than 5 mg/24 hours). Because the patient had two older sibs who had died in the neonatal period with similar clinical symptoms, these investigators thought that a "new" inborn error of metabolism was responsible for the increased excretion of methylmalonate.

Oberholzer and colleagues (15) also described two children with persistent metabolic acidosis, vomiting, and physical and mental developmental retardation who had abnormally high methylmalonic acid excretion. They showed that valine, protein, or propionate administration led to a fall in plasma pH and bicarbonate level, an increase in plasma ketones, and hypoglycemia. In addition, all three of these substances augmented the much increased methylmalonate excretion. In vitro tests with the two patients' leukocytes revealed abnormal propionate metabolism. When white cells were incubated with propionyl CoA and $NaHC^{14}O_3$, C^{14}-methylmalonate was formed in normal amounts, but no conversion to succinic, fumaric, or malic acids was found. These findings were confirmed by Morrow and colleagues (16) in cultured fibroblasts. These groups concluded that these patients had a block in the conversion of methylmalonate to succinate but did not distinguish between a defect in the racemase or mutase.

Rosenberg and colleagues (17) provided indirect evidence for a defect in the mutase enzyme in at least some of these patients by studies on a one year old boy with methylmalonic acidemia. They, too, found

that C^{14}-propionate conversion to $C^{14}O_2$ was negligible in leukocytes from this patient, but that succinate oxidation was intact. They concluded that the block was between propionate and succinate. Although this patient was not cbl-deficient, administration of large doses of parenteral CN-Cbl led to a fall in excretion of methylmalonate from 800-1200 mg/24 hours to 220-280 mg/24 hours, and an increase in the amount of propionate oxidation to CO_2 by intact leukocytes. In addition to providing indirect evidence for a mutase defect, this work established the concept of cbl-responsiveness in patientswith methylmalonic acidemia.

The demonstration of "cbl-dependency" allowed patients with methylmalonic acidemia to be divided into those that were cbl-responsive, i.e. showed decreased methylmalonate excretion with amelioration of symptoms after administration of pharmacologic doses of cbl despite the absence of cbl-deficiency (17-19), and those that were not (14,20,21). Other workers performed more detailed analyses on several of these patients with cbl-responsive methylmalonic acidemia in order to correlate clinical responsiveness (decreased methylmalonate excretion) with <u>in vitro</u> changes. Hsia and colleagues (22) obtained leukocytes from a cbl-responsive patient prior to cbl administration and showed that they failed to oxidize propionate or methylmalonate. However, oxidation was significantly increased in leukocytes after oral administration of cbl. Further, Morrow and colleagues (23) demonstrated that mutase activity in liver homogenates from a patient with cbl-responsive methylmalonic acidemia was increased to normal with the addition of saturating concentrations of Ado-Cbl to the incubation mixture. These groups concluded that methylmalonic acidemia was genetically heterogeneous with cbl-unresponsive patients having a defect in the mutase apoenzyme and the cbl-responsive

patients having a defect either in Ado-Cbl synthesis or in the mutase apoenzyme such that it had a decreased affinity for Ado-Cbl. Either of the latter two defects could, theoretically, be overcome with pharmacologic doses of cbl.

Recently, much work has been done to characterize further the cbl-responsive forms of methylmalonic acidemia (24-29). To date, four classes of mutants with defective cbl metabolism have been discovered. Mahoney and colleagues (24) first distinguished two groups of cbl mutants, designated cbl A and B, which were distinguished by their ability to synthesize Ado-Cbl. Both cbl A and B mutants were unable to synthesize Ado-Cbl in intact cells, but in a broken cell system cbl A mutants had normal Ado-Cbl synthesis. These workers proposed that cbl A mutants represented either a defect in cbl transport across the mitochondrial membrane or an abnormality of the cbl reductase system, and that cbl B mutants probably had a defective adenosyltransferase (see Figure 2). Further work by Mahoney and Rosenberg (25) identified a third class of cbl mutants, cbl C, in which there was defective formation of both Ado- and Me-Cbl. Fibroblasts from cbl A and B mutants accumulated no Ado-Cbl, but Me-Cbl metabolism and intracellular concntrations were normal. On the other hand, cbl C mutants had undetectable levels of either Ado- or Me-Cbl. This class of mutants was thought to explain the previously reported association of methylmalonic acidemia with homocystinemia and cystathionemia (26). Gravel and colleagues (27) in work with inactivated Sendai virus-mediated heterokaryons showed complementation between cbl A, B, and C mutants demonstrating a genetic heterogeneity corresponding to the biochemical heterogeneity.

Recently, evidence for a fourth class of cobalamin mutants, cbl D, has been described (28). Like cbl C mutants, the cbl D mutants lack mutase and methyltransferase activity, indicative of an absence of both Ado- and Me-Cbl; by complementation experiments, however, cbl C and D are genetically distinct. Neither class has been characterized biochemically; cbl uptake is normal, however, suggesting abnormalities in one or more early cytosolic steps in the intracellular pathway of cbl coenzyme synthesis.

A fifth class of *in vitro* cbl-responsive mutants has been found which does not complement with mutase apoenzyme mutants and has normal cbl metabolism. Willard and Rosenberg (29) have studied these two groups of apomutase mutants and have demonstrated that the cbl-responsive group, designated mut^-, have an altered K_m for Ado-Cbl (in the range of 10^{-4} M compared with 10^{-8} M for controls), reflecting decreased affinity of the enzyme for its cofactor. Mut^- mutants most likely represent base-pair substitutions which affect the Ado-Cbl binding site of methylmalonyl CoA mutase, but not the catalytic site. Mut^0 mutations, on the other hand, have no residual mutase activity regardless of cofactor concentrations, and may represent a deletion or nonsense mutation which results in no apoenzyme production or production of a non-functional protein.

Propionic Acidemia

In retrospect, the first patient with propionic acidemia was described by Childs and colleagues (30) in 1961. However, the increased excretion of propionic acid was not detected at that time and the disease was called "idiopathic hyperglycinemia" because of the increased glycine concentrations found in the patient's urine and blood. This patient presented at eighteen hours of life with vomiting, hyperpnea, acidosis, and ketonuria which persisted throughout the first week of life and resulted in a pyloromyotomy at age ten days. However, postoperatively the patient continued to vomit, failed to thrive, and had frequent infections; he was readmitted at eight weeks of age. At that time, he was found to have neutropenia, hypogammaglobulinemia, thrombocytopenia and purpura, and glycinuria. During the admission, it was demonstrated that the episodes of vomiting, lethargy, and ketosis could be terminated by withdrawal of oral feedings and intravenous administration of a glucose and electrolyte solution. Reintroduction of protein into the diet led to the symptoms of the acute illness with increased glycinuria and glycinemia. Challenge tests with the individual amino acids were well tolerated clinically except for leucine, methionine, isoleucine, threonine and valine, all of which resulted in vomiting and lethargy. The increased urinary glycine noted during these episodes led these workers to question whether the clinical and laboratory abnormalities were secondary to this increase. However, administration of sodium benzoate, which conjugates glycine to hippurate, did not prevent production of the typical metabolic acidosis, ketosis, vomiting, and lethargy in response to dietary protein loading despite a fall in

glycinuria and glycinemia. Further, other workers (31) demonstrated that infusion of glycine into one of these patients resulted in neutropenia, but none of the other clinical or biochemical abnormalities. Both groups concluded that the increased glycine was not the cause of the periodic illness, but was secondary to some other abnormality related to protein degradation.

Subsequent control of the illness in Childs' initial patient with a diet low in the offending amino acids, resulted also in a fall in glycine concentrations (32). Follow-up studies (33) in this patient two years after initiation of this special diet (age 4½) revealed that he had suffered a decreased frequency and severity of the periodic episodes of ketosis, had no thrombocytopenia or neutropenia, and had decreased infections. However, his growth was retarded with a height and weight well below the third percentile, and his mental and physical development were delayed, including the inability to speak and the inability to walk without help.

Although the primary defect remained obscure for many years, two "forms" of idiopathic ketotic hyperglycinemia were recognized. These two forms were called "acute" and "chronic" by Tanaka (3) and were differentiated by their age of onset and severity of symptoms. Hommes and colleagues (34) described a neonate who presented with hypotonia, areflexia, hyperventilation and grunting, and had a severe metabolic acidosis unresponsive to alkali therapy who died at five days of age. The early age of onset and the severity of the symptoms seen in this child are characteristic of Tanaka's "acute" group. At the other extreme, Brandt and colleagues (35) describe their experience with the sister of Childs' original patient. She had positive urinary ketones

at 44 hours of life and was started on a special formula low in the offending amino acids. She was followed to age eight at which time she had evidence of only mild osteoporosis, but had normal height and weight, no thrombocytopenia, anemia, or neutropenia, and an IQ of 125. The mild nature of this child's disease is characteristic of Tanaka's "chronic" group. In between these two extremes were children like Childs' original patient who survived the neonatal period but suffered severe mental and physical retardation. This clinical heterogeneity was paralleled by a biochemical heterogeneity. For example, infants who were shown to have propionic acidemia were originally described without hyperglycinemia (34) and without ketosis (36). This clinical and biochemical heterogeneity led workers to postulate that the spectrum of disease in patients with propionic acidemia was secondary to genetic heterogeneity at the locus which controlled the primary defect in protein metabolism.

In 1968, Rosenberg and colleagues (17) noticed several similarities between patients with "idiopathic hyperglycinemia" and those with methylmalonic acidemia including protein induced ketosis and acidosis, long-chain ketonuria, and intermittent hyperglycinemia. These similarities led these workers to postulate that the defect in idiopathic hyperglycinemia was along the pathway of propionate metabolism, but since no methylmalonate was found in the urine, the block must be proximal to the production of methylmalonyl CoA. The finding by other groups (34,37) of increased propionic acid in the urine and blood of patients with idiopathic hyperglycinemia suggested that the primary defect in these children was a decrease in propionyl CoA carboxylase (PCC) activity. Other indirect evidnece for a defect in the PCC enzyme was found at autopsy of the patient described by Hommes at which a

fatty liver was found. Analysis of the stored triglycerides revealed unusual C_{15} and C_{17} straight chain fatty acids. These odd-numbered fatty acids were explained by a defect in the PCC enzyme resulting in a secondary increase in propionyl CoA which could serve as a substitute for acetyl CoA as a primer in fatty acid biosynthesis. In addition, products of alternate pathways of propionyl CoA metabolism, butanone (31) and propionylglycine (38), have been identified.

More direct evidence for PCC deficiency in patients with ketotic hyperglycinemia came first from Hsia and colleagues (39) who demonstrated that peripheral leukocytes from a patient with propionic acidemia could not oxidize propionate to CO_2 as well as leukocytes from controls, although oxidation of methylmalonate was normal. Ando and colleagues (38), in addition to confirming these in vitro studies in fibroblasts from a patient with propionic acidemia, demonstrated that in vivo conversion of propionate to CO_2 was defective. Synthesis of radiolabeled CoA derivatives of propionate and methylmalonate finally allowed direct measurement of PCC activity. Activity in leukocytes and cultured fibroblasts were reported by several groups (38,40,41) who found less than 2% of normal activity in each of three patients with propionic acidemia.

Analagous to methylmalonyl CoA mutase, propionyl CoA carboxylase requires a coenzyme, biotin, for activity. Barnes and colleagues (42) described a patient with propionic acidemia who was not biotin deficient, but who responded clinically and biochemically to pharmacologic doses of biotin (10 mg/ 24 hours). This patient was maintained on a 0.1 gram protein/ kg diet and then given an isoleucine load, 0.1 g/ kg, which resulted in lethargy with metabolic acidosis, ketosis, hyperglycinemia and increased plasma propionate. This was repeated after giving 5 mg

of biotin twice daily for five days. The fasting plasma propionate value, as well as the height and duration of the propionate response to isoleucine loading was significantly decreased as was the appearance of clinical symptoms. On the other hand, the patient described by Hsia and colleagues (40) did not respond to biotin supplements. Thus, as was described for methylmalonic acidemia, propionic acidemia has both vitamin-responsive and vitamin-unresponsive forms. The biotin is linked to the apoenzyme of PCC through an ϵ-amino group of lysine by an apoenzyme-biotin ligase. An altered ligase or a PCC attachment site with decreased affinity for biotin which could be overcome with increased biotin are likely causes for the biotin-responsive variants of propionic acidemia. Biotin-unresponsive mutants most likely represent a mutation resulting in either a defective apoenzyme or absent enzyme production.

The clinical heterogeneity, called "acute" and "chronic" by Tanaka, was not a function of this biotin-responsiveness, nor was it the result of the amount of residual PCC activity since some patients with the "chronic" form had less residual activity than those with the "acute" (43). Other work with Sendai virus-induced heterokaryons demonstrated two major complementation groups, designated pcc A and pcc C (44). However, this genetic heterogeneity cannot explain the clinical differences since each complementation group is clinically heterogeneous. In fact, within the same family with two affected sibs, one child had severe coma leading to death, while the other was asymptomatic with normal intelligence (45). Tanaka concluded that the difference in clinical expression depends upon the balance between production and disposal of propionate, which depends, in turn, on the diet, and on the existence of alternate pathways of removal, probably during a critical period of brain growth in

the neonate (3).

α-methylacetoacetic Acidemia

In 1971, Daum and colleagues (46) described a child who presented at 22 months of age with symptoms compatible with the known disorders of propionate metabolism except that he ahd normal propionate and methylmalonate concentrations in the body fluids. However, this child excreted abnormal amounts of two other organic acids, α-methylacetoacetic and α-methyl-β-hydroxybutyric acids. These workers suggested that this child had a new disorder of amino acid metabolism, which was later shown to be β-ketothiolase deficiency. This patient's clinical course was mild with only five episodes of ketoacidosis before age five years, and normal physical and mental development.

Other groups have subsequently described additional children with these clinical and biochemical characteristics who had more severe clinical findings. Keating and colleagues (47) described a child with projectile vomiting who underwent pyloromyotomy but continued to vomit and exhibit failure to thrive postoperatively. At age three months, he presented with lethargy, thrombocytopenia, hyperglycinemia and glycinuria, osteoporosis, hyperammonemia and long-chain ketonuria, all of which responded to a restricted protein diet. Isoleucine challenge after stabilization on this diet resulted in the recurrence of the above symptoms and findings.

Daum and colleagues (48) reviewed the two pedigrees from their and Keating's patient and determined that, like propionic and methylmalonic acidemias, this new disorder could present anywhere in a spectrum of clinical and biochemical severity from mild intermittent episodes of ketoacidosis with normal growth and development to severe recurrent

episodes characterized by intermittent vomiting, metabolic acidosis, coma and the other biochemical features of the ketotic hyperglycinemia syndrome. These workers suspected that the abnormal clinical and biochemical findings were related to protein catabolism and demonstrated significant increases in the abnormal urinary metabolites in response to isoleucine loading. They proposed that the combination of normal propionate and methylmalonate concentrations with the buildup of the two substances proximal to the β-ketothiolase reaction, pointed to a defect in that enzyme as a cause of this new disorder. They proposed that the appearance of both of the abnormal metabolites was due to an equilibrium of the reversible interconversion reaction between the two compounds, the balance of which, like the acetoacetate/β-hydroxybutyrate ratio, depends on the NAD/NADH ratio in the cell.

The localization of the defect was demonstrated more directly by Hillman and colleagues (49) who described a child who presented in the first week of life with symptoms of the ketotic hyperglycinemia syndrome. Metabolism of propionate and methylmalonate in leukocytes and cultured fibroblasts from this patient were normal. However, oxidation of isoleucine to CO_2 was less than 40% of controls. They concluded that this finding, along with the appearance of butanone in the urine (which was thought to be a metabolite of α-methylacetoacetic acid), localized the defect to the β-ketothiolase enzyme.

Gompertz and colleagues (50) described a child who presented at seven years of age having survived childhood with only complaints of frequent episodes of abdominal pain, headaches and vomiting. They measured tiglyl CoA-dependent incorporation of tritiated water into

propionate in leukocytes of this patient and found a significant decrease in activity compared to control cells. This localized the defect to one of two enzymes preceeding propionyl CoA in the pathway of isoleucine degradation.

Urea Cycle

The primary defects in the pathways demonstrated above must, in some way, affect the turnover of ammonia in patients with these organic acidemias and lead to hyperammonemia. This effect may be an increase in ammonia production, i.e. negative nitrogen balance, decreased removal, i.e. impairment of the urea cycle, or both. In order to understand how a defect in organic acid metabolism may lead to hyperammonemia by this latter mechanism, it is first necessary to examine the urea cycle, its enzymes, and the control of its activity.

Ammonia is formed primarily as the end product of amino and nucleic acid catabolism from the amide groups of glutamine and asparagine, the amine groups of glutamate and aspartate, and the amino groups of adenine, guanine and their derivatives. In addition, microbial deaminases and ureases found in the normal flora of the large intestine cleave amino acids and urea to ammonia which is absorbed by the gut and contributes to the blood ammonia pool. To prevent the buildup of ammonia and its consequent neurological toxicity, ammonia is removed via several pathways. First, ammonia is used in the synthesis of amino acids by reversal of several of the deamination reactions. In addition, ammonia can be used in purine, pyrimidine and porphyrin synthesis via incorporation into glutamine, carbamyl phosphate, and glycine. Lastly, and most important, ammonia is converted into nitrogen containing organic compounds, urea in mammals and uric acid in birds and reptiles, which are excreted from the body.

Urea is formed in mammals via the urea cycle in which two moles of ammonia and one mole of carbon dioxide are consumed in the production

of one mole of urea through a pathway of intermediates. That the liver is the major site of urea formation has been known since 1924 when Bollman and colleagues (51) demonstrated that removal of the liver in experimental dogs led to a decreased urea concentration in the blood.

In 1932, Krebs and Henseleit (52) began the work in which the initial biochemical steps of the urea cycle were discovered. They demonstrated that urea could be synthesized in vitro in rat liver slices from ammonia and carbon dioxide (in the form of bicarbonate) and this began a search for other compounds that could act as a nitrogen donor to urea. One of the amino acids tested was ornithine. They found that one molecule of ornithine stimulated the formation of 20 molecules of urea when ammonia was present. However, the ornithine was not consumed in the process and the total urea nitrogen could be accounted for by the disappearing ammonia. These puzzling kinetics remained a mystery until the discovery of a pathway in which ornithine, carbon dioxide, and ammonia combine to form arginine. Along with the long known presence of an enzyme, arginase, which catalyzes the reaction in which arginine is split into ornithine and urea, the original ornithine cycle was proposed. (see Figure 3)

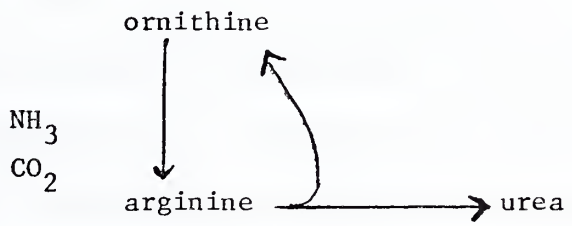

Figure 3. The original ornithine cycle as proposed by Krebs and Henseleit in 1932

Further work has elucidated the urea cycle as we now know it (see Figure 4), with five reactions catalyzed by the five major enzymes of the cycle. The initial step in the urea cycle is the fixation of one mole of ammonia and one mole of carbon dioxide to form a high energy intermediate, carbamyl phosphate. This reaction requires ATP and is catalyzed by CPS I, an enzyme which uses ammonia as the nitrogen donor and which is found primarily in the mitochondrial matrix of liver cells. This enzyme is distinct from CPS II, an enzyme of pyrimidine biosynthesis found in the cytosol of most cells and which uses glutamine as a nitrogen donor (53). CPS I is a dimer of MW 250,000; each subunit is identical with MW of 125,000 (54). This reaction requires, in addition to the substrates mentioned above, Mg^{+2} (needed for ATP binding) and K^{+} (increases the affinity of the enzyme for its substrates) (55). Two moles of ATP are consumed per mole of carbamyl phosphate formed: one mole is consumed in the dehydration of bicarbonate and yields a high energy intermediate which adds to ammonia to form another high energy intermediate thought to be carbanyl phosphate; and a second ATP that donates a phosphoryl group to carbamate to yield carbamyl phosphate (56).

An important discovery about the CPS I reaction was made by Grisolia and Cohen (57,58) when they demonstrated that this reaction required a glutamate derivative as an activator. Their initial experiments used carbamyl glutamate, but six years later, Hall and colleagues (59) identified N-acetylglutamate (NAG) as the naturally occurring cofactor for carbamyl phosphate synthesis in mammalian liver. This was confirmed by work which demonstrated the presence of NAG synthetase, an enzyme found in the mitochondrial matrix which catalyzes the formation of NAG

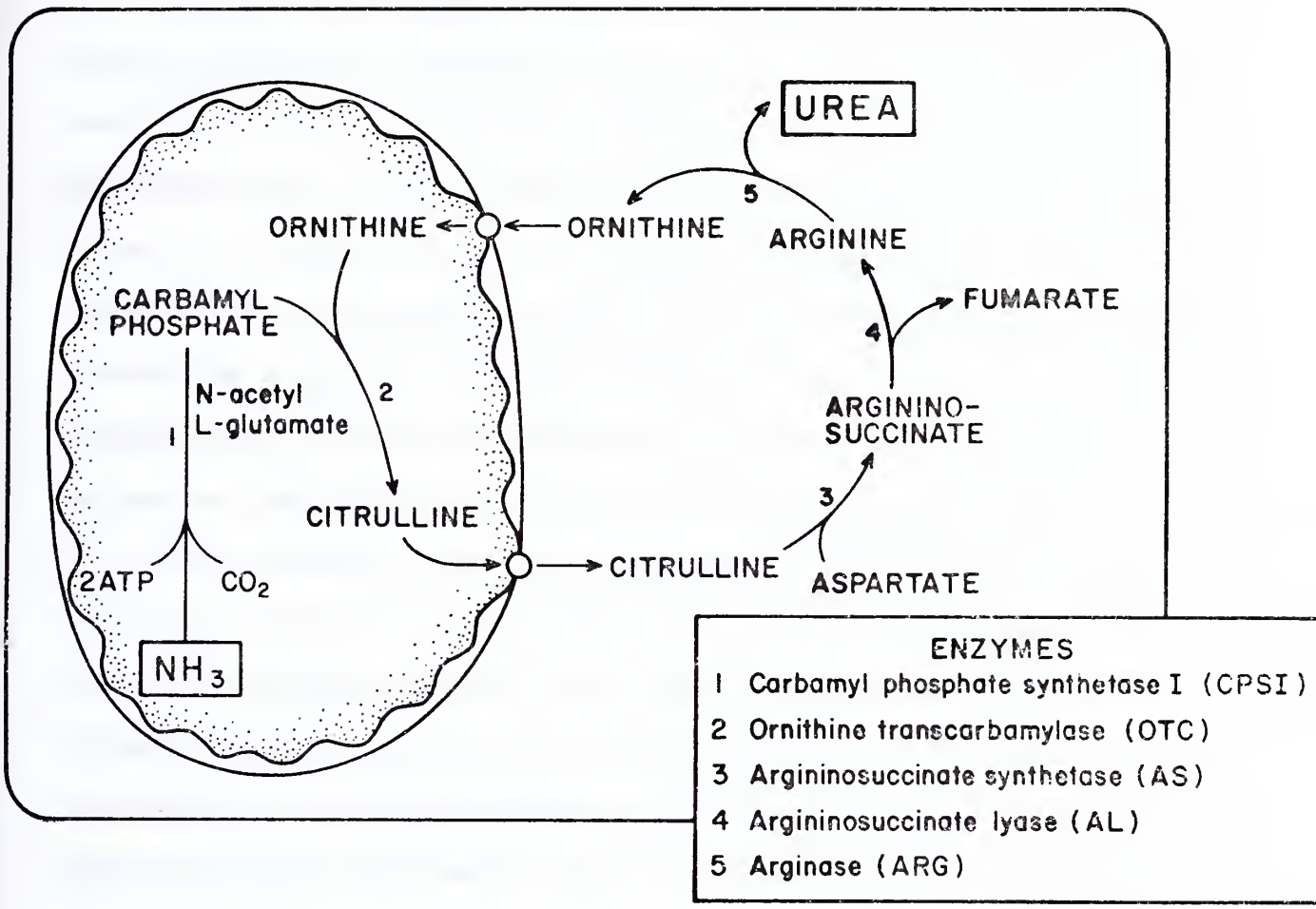

Figure 4. The urea cycle. The mechanism of ammonia detoxification in mammals involves the condensation of nitrogens from ammonia and aspartate with CO_2 to form urea through the five enzyme cycle shown above.

from L-glutamate and acetyl CoA and which is stimulated by L-arginine (60). NAG causes a conformational change in CPS I which results in activation of the enzyme (54).

Ornithine transcarbamylase (OTC) is an intramitochondrial enzyme which catalyzes the condensation reaction between carbamyl phosphate and ornithine to form citrulline (61). OTC from bovine liver is a trimer of MW 108,000; each subunit is identical and of MW 36,000 (62). Although trimers are unusual in mammalian systems, the demonstration of three binding sites for carbamyl phosphate (one per subunit) with three different dissociation constants, substantiates this earlier finding (63). Citrulline formed in this reaction is transported from the mitochondria to the cytosol on a specific carrier which is not energy dependent (64).

Cohen and Hayano first demonstrated that citrulline could be converted to arginine in rat liver slices (65). Ratner (66) demonstrated that Mg^{+2} is required and that either aspartate or glutamate (in the presence of oxaloacetate) acted as the nitrogen donor. Much work was done in elucidating the enzyme system responsible for this ammonium transfer. Ratner and Pappas (67) demonstrated that two enzymes were required: the first catalyzed a condensation between citrulline and aspartate to yield an intermediate; the second cleaved this intermediate to arginine and malic acid. This intermediate was identified as argininosuccinate (68) and the reaction required one mole of ATP per mole of product formed (69). The enzyme that catalyzes this reaction is argininosuccinate synthetase. It is localized in the cytosol and has a MW of 180,000 with four identical subunits each of MW 45,000 (70). Associated with the synthetase enzyme is a pyrophosphatase which removes the inorganic phosphate formed and pulls

the condensation reaction in the direction of product formation (68).

Argininosuccinase (argininosuccinic acid lyase) cleaves argininosuccinate to arginine and fumaric acid (71). (The finding of malic acid by earlier groups (67) was believed to be secondary to the fumarases present in the acetone-dried liver preparations used.) Argininosuccinase is a tetramer of MW 202,000. It contains four identical subunits weighing 50,000 daltons each (72), with one catalytic site per polypeptide chain (73).

Finally, arginase splits arginine into urea and ornithine. Arginase is a Mn^{+2}-requiring enzyme of MW 118,000 with four identical subunits weighing 30,500 daltons each (77). The ornithine formed enters the mitochondria via an energy-dependent carrier which is specific for ornithine^{+} but not arginine^{+} or lysine^{+}, and is stereospecific for L-ornithine (67).

Regulation of the Urea Cycle

The exact mechanisms by which urea production is controlled are not known despite a considerable amount of work in this area. That a control mechanism does exist has been known since 1905 when Folin (75) demonstrated that urea production in normal adults was proportional to the intake of protein. Urea excretion fell within a day when a low protein diet in the form of a "starch and cream diet" (400 g pure starch and 300 ml of cream) was fed to his subjects; urea production increased within a day when a regular diet was reinstituted.

The controversy over the control of urea production has been waged between those investigators who believe that control is mediated largely by changes in enzyme activities and those who believe that control is mediated by changes in substrate concentrations. Nuzum and Snodgrass (76)

demonstrated that an increase in the protein content of a monkey's diet from 1.6 to 16 g protein/ kg resulted in a two to three fold increase in the activity of all urea cycle enzymes in liver homogenates in vitro. Further, Schimke (77) showed that this increase in enzyme activity was linearly related to the increase in protein, and concluded that the increased urea production was due to increased enzyme activity. Schimke isolated OTC and arginase from rats fed diets containing 15% and 60% protein and showed that both enzymes had the same pH optima, K_m's for substrates, MW, and turnover number regardless of the protein intake. He concluded that the increased activity of the urea cycle enzymes was due to an increased number of protein molecules and not to a change in the kinetic parameters of the molecules already present. The activities of the non-urea cycle enzymes measured such as glutamate dehydrogenase were not elevated at higher protein concentrations, so that the increase in urea cycle enzyme activities was not a non-specific increase due to more available amino acids for protein synthesis. Schimke followed the time course of the increase in activity and demonstrated that it was completed within four days for OTC, CPS I, and argininosuccinase and within eight days for arginase.

Other workers argued that the augmentation of urea production must depend on the substrate load, not on the activity of the urea cycle enzymes, since the rate of urea excretion demonstrated by Folin changed more rapidly than the change in urea cycle enzyme activity described by Schimke. In support of this concept is the work by McGivan and colleagues (78) in which the rate of citrulline synthesis and the activities of the urea cycle enzymes were measured under different dietary conditions. In rats

fed a high protein diet (egg whites), the rate of citrulline synthesis was increased 20 fold compared to rats fed 20% glucose. However, the maximal activities of the urea cycle enzymes did not differ greatly under these two dietary conditions. The disparity between these results and those of Nuzum and Snodgrass (76) and Schimke (77) remains unexplained.

Tatibana and Shigsada (79) proposed that the regulation of urea production is accomplished via the acetylglutamate-arginine system, composed of two enzymes, CPS I and NAG synthetase, and their two activators, NAG and arginine. They believe that glutamate and arginine are representative of the amino acid pool and that increases in these two amino acids reflect increased protein catabolism. Arginine stimulates the production of NAG (which is accomplished because of the increased amount of glutamate); NAG, in turn, stimulates the production of carbamyl phosphate. (In addition, arginine may supply ornithine, a critical rate-limiting substance.) In experiments with protein loaded mice, Shigesada and Tatibana (80) demonstrated that the NAG concnetration in mitochondria increased in parallel with the increased protein in the diet. When the percentage of the protein in the diet was increased from zero to 60%, the NAG concentration increased from 15 to 40 nmoles/ g liver tissue. In addition, when C^{14}-glutamate was injected into three mice who were sacrificed 30 minutes, two hours, and nine hours later, NAG isolated from liver samples had 3900, 5900, and 260 cpm per g wet weight liver, respectively. This indicates that significant synthesis of NAG occurs within thirty minutes, a response quick enough to explain the increased urea production described by Folin. In further support of this hypothesis, these workers demonstrated that the increase in NAG paralleled the increase excretion

of urea after protein loading (79).

Further elaboration of this mechanism was reported in recent work by Stewart and Walser (81). Rats were sacrificed at certain time intervals after an intraperitoneal injection of a 1.5 g/ kg mixture of amino acids. Glutamate and NAG concentrations, as well as CPS I activity peaked five to fifteen minutes after injection. Surprisingly, this effect was reproducible and equal with or without arginine in the mixture, indicating that the increase in NAG was not secondary to an activation of NAG synthetase by arginine. These authors concluded that control of ureagenesis was, at least in part, via control of glutamate concentrations. According to this notion, protein loading results in increased concentrations of glutamate which forms NAG and activates CPS I. At doses of amino acids of 3 g/ kg, CPS I activity was maximally activated and reached the same value seen in disrupted mitochondria with saturating concentrations of NAG. Moreover, at doses of amino acids from 3-5 g/ kg, the autoregulatory mechanism became saturated and hyperammonemia supervened.

Other authors have examined other steps in the urea cycle as possible control sites. Krebs and colleagues (82) suggest that since the K_m of ornithine for OTC is 1.4 mM, the normal extracellular concentration of ornithine (0.3 mM) makes it likely that ornithine is rate-limiting *in vivo*. This may explain the reported stimulatory effect of ornithine on the urea cycle (52), as well as recent work reported by Batshaw and colleagues (83) in which arginine infusions (as a source of ornithine) resulted in a fall in blood ammonia in infants with argininosuccinic aciduria.

Finally, it has been suggested that ATP may be the rate-limiting reagent (84). In work in which animals were exposed to an anoxic

challenge, the concentration of ATP was lowered to less than 10% of normal; this was accompanied by a significant rise in blood ammonia.

Thus, the controversy continues between the "enzyme hypothesis" and the "substrate hypothesis". Changes in maximal enzyme activity as measured *in vitro* do not occur fast enough to account for the increased urea production, but this *in vitro* work may not accurately reflect the *in vivo* situation. Increased substrate concentrations may lead to increased urea production if enzymes are not fully saturated, but the *in vivo* effect of changes in substrate concentration awaits the accurate determination of intramitochondrial concentrations and comparison to K_m values. It is likely that a system as complex as the urea cycle is regulated by changes in both substrate concentrations and enzyme activities.

The Organic Acidemias and Hyperammonemia

Several biochemical abnormalities including acidosis, hyperglycinemia, hypoglycemia and hyperammonemia are common to each of the inherited organic acidemias and are thought to occur secondary to the primary defect in organic acid metabolism. Although hyperammonemia has not been considered a constant finding in all of these disorders, it is commonly found in infants with propionic acidemia (85,86), methylmalonic acidemia (20,87,88), and α-methylacetoacetic acidemia (47), and may contribute to the metabolic encephalopathy which so often occurs in affected newborns and young children. The precise mechanism of this hyperammonemia has not yet been identified, but much work has been done in an attempt to link the primary enzymatic defect with this secondary metabolic derangement.

Rosenberg and colleagues demonstrated that isoleucine loading in two patients with propionic acidemia resulted in a significant increase in blood ammonia values which were correlated with rises in serum propionate (89). This work suggested that the buildup of a metabolite or precursor of propionate in the pathway of isoleucine degradation interferes with ammonia detoxification and leads to hyperammonemia. These workers proposed that this metabolite could directly inhibit one of the urea cycle enzymes or damage the mitochondria and interfere with the delivery of one of the substrates of these enzymes, e.g. decrease ornithine or citrulline transport across the mitochondrial membrane.

Since the major pathway of propionate metabolism is located within the mitochondrion, the most likely site of action for any inhibitor would be on that portion of the urea cycle which occurs intramitochondrially. This hypothesis was confirmed in work by Glasgow and Chase (90)

who demonstrated that 5 mM concentrations of propionate inhibited ureagenesis *in vitro* by 40 % in rat liver slices when ammonia, but not citrulline or aspartate, was the substrate. Propionate, however, had no effect on either CPS I or OTC activities when measured in rat liver homogenates. They concluded that this inhibition of ureagenesis (as well as the hyperammonemia seen in patients with the organic acidemias) was due either to a direct inhibition of CPS I or OTC by a metabolite of propionate, or to inhibition of citrulline or ornithine transport across the mitochondrial membrane, or to depletion of one of the substrates for the CPS I reaction.

Several intramitochondrial sites for action of such a metabolite have been proposed. Some workers have presented evidence from *in vitro* experiments which favors the third possibility, namely that a decreased availability of one of the substrates of the CPS I reaction leads to the hyperammonemia. Cathelineau and colleagues (91) demonstrated that propionate decreased carbamyl phosphate concentration, citrullinogenesis, and ATP content. They proposed that this decreased ATP concentration and its consequent interference with CPS I activity could lead to hyperammonemia. However, Stewart and Walser (92) failed to find a decrease in ATP levels in mitochondria isolated from rats injected with 20 mmoles/ kg propionate or methylmalonate, doses which caused significant increases in blood ammonia.

Other workers have focused on the role of N-acetylglutamate (NAG), the required allosteric activator of CPS I. Shigesada and Tatibana (60) initially described inhibtion of NAG synthetase by propionyl CoA, and Bachman (93) first proposed that such inhibition may account for the hyperammonemia seen in infants with propionic and methylmalonic acidemias. If significant amounts of propionyl CoA build up behind the enzymatic

blocks in these disorders, NAG availability and, hence, CPS I activity could decrease, leading to hyperammonemia.

Coude and colleagues (94) working in vitro with rat liver mitochondria confirmed this inhibition and analyzed its kinetics extensively. They demonstrated that propionyl CoA was a competitive inhibitor of NAG synthetase and that the K_I is 0.71 mM, well within the range of propionate concnetrations observed in patients with these disorders.

Recently, Stewart and Walser (92), working with an animal model for these disorders, have demonstrated that rats injected with 10-20 mmoles/ kg propionate or 20 mmoles/ kg methylmalonate and 1.5 g/ kg of an amino acid mixture developed hyperammonemia. Furthermore, although ATP levels were unchanged in mitochondria isolated from these rats, NAG content and CPS I activity were significantly decreased. Moreover, they demonstrated that acetyl CoA concentrations were significantly decreased while acyl CoA's (presumably propionyl and methylmalonyl CoA's) were increased. They suggested that NAG availability was decreased either because of competitive inhibition of NAG synthetase by propionyl CoA or from depletion of acetyl CoA. Thus, NAG depletion was highlighted as the putative mechanism for the hyperammonemia seen in patients with the inherited organic acidemias. However, these workers failed to show any decrease in NAG concentrations with injections of 10 mmoles/ kg methylmalonate, a dose which led to significant decreases in acetyl CoA and an increase in blood ammonia. In addition, methylmalonyl CoA has been shown not to significantly inhibit NAG synthetase (60).

Alternatively, propionyl CoA may act as a substrate for NAG synthetase leading to the production of N-propionylglutamate (NPG), a compound less effective as an activator for CPS I, which could compete with NAG for its

CPS I binding site. That NPG is a weak activator of CPS I was confirmed by Coude and colleagues who demonstrated a K_a for NPG of 1.1 mM, ten times the value obtained for NAG. A buildup of propionyl CoA, therefore, could result in the production of NPG instead of NAG, and consequently CPS I activation would be decreased and hyperammonemia would occur.

Part II: The Organic Acidemias and Hyperammonemia: Experimental

Introduction

All of the work summarized in the Review section suggests that organic acid accumulation impairs the intramitochondrial synthesis of carbamyl phosphate by decreasing the concentrations of CPS I substrates (ATP) or activators (NAG), or by favoring the synthesis of a much poorer activator (NPG). The present studies were undertaken to examine another hypothesis, namely that a metabolite in the pathway of branched-chain amino acid degradation directly inhibits one of the urea cycle enzymes. I have demonstrated that several acyl CoA's significantly inhibit CPS I activity in rat liver homogenates, disrupted mitochondria, and purified enzyme, as well as in human liver homogenates.

Materials and Methods

125 g female rats were purchased from Camm Laboratories in Wayne, N.J. and were fed a standard commercial diet. Rats were sacrificed by decapitation and livers were quickly excised. Liver for homogenates was quick frozen in dry ice and stored at -90°C until needed. Human liver was obtained from autopsy specimens within one hour of death, quick frozen and stored. 5% (w/v) homogenates for CPS I and arginase assays and 0.5% homogenates for OTC assays were prepared daily from frozen liver which was weighed wet and homogenized in distilled water.

Preparation of Mitochondria

Mitochondria were prepared from fresh rat liver using the method of differential centrifugation of Loewenstein (95) as modified by Fenton and colleagues (96). Liver from decapitated rats was excised, weighed wet, and suspended in a solution (SET solution) containing 0.25 M sucrose, 17 mM Tris-HCl and 1 mM Na-EGTA pH 7.4 to make it 15% weight/ volume. The liver was minced with scissors and then homogenized with six to nine passes at 2100 rpm. The homogenate was centrifuged at 5000 rpm for one minute to remove the nuclear pellet and whole cells. The supernatant was recentrifuged for two minutes at 12,000 rpm. The pellet containing mitochondria and lysosomes was suspended in SET solution, 1 ml per gram starting liver. An equal volume of digitonin solution (1.75 mg/ml in SET solution), was added and the mixture gently agitated at 4°C for two minutes. This was then diluted to 5 ml/ gram starting liver and spun at 12,500 rpm for 4.5 minutes. The supernatant was removed and the

pellet was washed twice in SET solution, 2 ml/ gram starting liver and centrifuged for 5.5 minutes. The yield from this procedure was generally about 1 g mitochondria per 100 g liver.

Purified Enzyme

Purified CPS I was obtained as a gift from Dr. Carol Lusty (97). The enzyme was stored frozen at -90°C in a buffer containing 50 mM Tris- acetate (pH 8.0), 2 M ammonium sulfate, 0.1 M sodium acetate, 0.01 M magnesium acetate, and 2 mM dithioerythritol. The frozen sample was thawed just prior to use and the cations were removed by the technique of Sephadex centrifugation as described by Lusty (98). G-50 (fine) Sephadex was equilibrated with 0.05 M Tris-acetate buffer, pH 8.0 (4°C) and a column was poured into a one ml tuberculin-type syringe. The column was washed with two volumes of this buffer and then packed by centrifugation for three minutes at 100 X g. A sample of enzyme preparation (10-100 microliters) was brought to a final volume of 100 microliters with buffer and placed on the top of the column. The enzyme was collected by centrifugation for 3 minutes at 100 X g and then diluted in buffer containing 50% glycerol. No dithioerythritol or dithiothreitol was added to the enzyme at the end of the procedure unless noted below.

Hepatic Enzyme Assays

CPS I activity in liver homogenates was determined by the colorimetric measurement of citrulline synthesis using a modification of the method of Brown and Cohen (99). The final assay volume was 0.1 ml containing 0.01 ml 5% homogenate, 10 mM NAG, 20 mM ATP, 20 mM $MgCl_2$, and 50 mM NH_4HCO_3. The latter three reagents were added as part of a mix which was added to initiate the reaction and also contained 40 mM Tris-HCl buffer,

(pH 7.5), 9 mM ornithine, and 0.5 units purified bovine OTC. The final reaction was ay pH 7.2. Reaction mixtures were incubated for ten minutes at 37°C; reactions were terminated by the addition of 0.1 ml 10% trichloroacetic acid. A boiled enzyme blank, zero-time blank, and blank without NAG gave similar results and hence a zero-time blank was used. Color development was carried out using a modification of the method of Archibald (100). Each tube contained 0.15 ml from the assay mixture, 0.85 ml of water, and 3 ml of a solution which contained 1 volume diacetylmonoxime (200 mg in 50 ml 7.5% NaCl) and 2 volumes acid solution (1.85 g antipyrine and 1.25 g ferrous ammonium sulfate in 250 ml water added to 125 ml concentrated phosphoric acid and 125 ml concentrated sulfuric acid). Citrulline standards of 2.5 to 10 micrograms were made from a solution which contained 15 mg% citrulline in 0.1 N HCl diluted 1:2 with water. All tubes were put into a boiling water bath for 30 minutes and then cooled. A_{464} was read for the sample tubes and compared to standard values. Inhibitors were added just prior ro the initiation of the assay. All reactions were run in duplicate with control tubes in each study. Control activities in rat liver homogenates ranged from 2.0-2.8 umoles/hr/mg protein and in human liver homogenates from 0.8-1.0 umoles/hr/mg, both in agreement with data from other authors (101).

Measurement of CPS I activity in isolated mitochondria was performed using the above assay conditions after gentle sonification (setting 1 on a Branson cell disrupter 185) of the mitochondria at 4°C for 30 seconds. Final pH was 7.2. A zero-time blank was used. Control activities for rat liver mitochondria assayed at 37°C were 9.6-12 umoles/hr/mg mitochondrial protein, which is in agreement with other authors (101).

Control activity at 30°C was 4.0 umoles/hr/mg. This temperature dependence is in agreement with other work (58).

CPS I activity in purified enzyme preparations was determined using the above assay conditions. Protein content was determined from the A_{280}, assuming an OD reading of 1.0 for a solution with a concnetration of 1 mg/ml. Control activity was 120 umoles/hr/mg, in agreement with other work (97).

OTC activity in rat liver homogenates was measured by the colorimetric determination of citrulline synthesis as described by Nuzum and Snodgrass (101). The final assay volume was 0.1 ml containing 0.01 ml of 0.05% homogenate, 10 mM ornithine and 10 mM carbamyl phosphate. The mixture was incubated for 15 minutes at 37°C and the reaction was terminated by the addition of 0.1 ml 10% trichloroacetic acid. Color development was carried out as described above except that each tube contained 0.9 ml of water and 0.1 ml from the assay. Control activity was 67 umoles/hr/mg, in agreement with other authors (101).

Arginase activity in rat liver homogenates was measured using the colorimetric determination of urea synthesis by a modification of the method of Nuzum and Snodgrass (101). 0.01 ml of a 5% liver homogenate was preincubated with 0.09 ml 1% bovine serum albumin, 0.5 ml 0.07 M glycine, 0.05 ml 0.3 M NaCl, and 0.1 ml 0.1 M $MnCl_2$ for one hour at 37°C. The assay mixture contained 0.2 ml 375 mM arginine, pH 9.5, and 0.1 ml of the above incubation mix. This mix was assayed for 10 minutes at 37°C and the reaction was terminated by the addition of 0.1 ml of 20% trichloroacetic acid. Color development was carried out by mixing 0.2 ml of the assay mixture, 2 ml of an acid reagent containing 18 ml of

concentrated sulfuric acid, 54 ml of concentrated phosphoric acid and 0.2 ml 0.1 M $FeCl_3$, and 0.1 ml 3% 1-phenyl-1,2-popane dione-2-oxime in 95% ethanol. Urea standards were prepared from a stock solution containing 25 mg% urea in 0.1 N HCl. All tubes were placed in a boiling water bath for one hour in the dark and then cooled. A_{540} was read for each sample and compared to standards. Control activity was 500 umoles urea/hr/mg, in agreement with other authors (101).

Protein was determined by the method of Lowry (102), unless as otherwise noted above. Diluted samples were added to 5 ml of a solution containing 0.5 ml 1% $CuSO_4$, 0.5 ml 2% sodium tartate, and 50 ml 2% Na_2CO_3 in 0.1 N NaOH. This mixture was incubated for 10 minutes at room temperature and then 0.5 ml 2 N Phenol Reagent (Fisher), diluted 1:1 with water, was added and incubated for 30 minutes more at room temperature. A_{625} was read for each sample and compared to known concentrations of bovine serum albumin.

Inhibitors

Potential inhibitors selected for study were organic acids in the pathway of isoleucine degradation, as well as their CoA esters. Inhibitors were added just prior to the initiation of the assay unless specified. The pH of the final assay mixture was measured with each inhibitor and there was no significant difference from control. The organic acids and isoleucine were purchased from Sigma. Methylcitrate was a gift from Dr. Kay Tanaka. The CoA esters were purchased from PL Biochemicals with the exception of methylmalonyl CoA which was the gift of Dr. Wayne Fenton and propionyl CoA which was synthesized as described below.

Propionyl CoA was synthesized from propionic anhydride (Eastman)

and Coenzyme A (PL Biochemicals) following a modification of the method of Flavin and Ochoa (103). 500 mg of coenzyme A was added to 30 ml of 1 M ammonium bicarbonate (pH 9.0), 7 ml of propionic anhydride and 33 ml of ethanol. The propionyl CoA formed was extracted three times with ether, adding an equal volume of ether and water. The excess ether was removed by vacuum suction extractions. After the final extraction, the propionyl CoA was lyophilized and resuspended in 4 ml of water. The concentration of propionyl CoA in the final solution was calculated by measurement of the A_{232}, using an extinction coefficient of 7.85.

Calculation of Enzyme Inhibition

Calculations of enzyme inhibition were complicated by the fact that the acyl CoA's inhibited the colorimetric reactions used to determine CPS I, OTC, and arginase activities. To correct for suppression of color development, propionyl or other CoA's were added to assay blanks (i.e. without homogenate), carried through the assay procedure, added to standard concentrations of citrulline and urea, and finally carried through the color development process. These were compared to similar standards containing no CoA esters to obtain an estimate of color suppression. Enzyme inhibition was calculated using the formula (Observed activity with inhibitor) = (Control activity) X (1 - fraction of enzyme inhibition) X (1 - fraction color suppression), solving for fraction of enzyme inhibition. Figure 5 demonstrates the effect of propionyl CoA at concentrations of 0, 1, 3, and 8 mM in suppressing colorimetric determination of citrulline. At each concentration of propionyl CoA, the fractional residual color developed at OD_{464} (i.e. (1 - fraction color suppression))

is equal for all concentrations of citrulline: for 1 mM propionyl CoA, 0.95; 3 mM, 0.85; and 8 mM, 0.65.

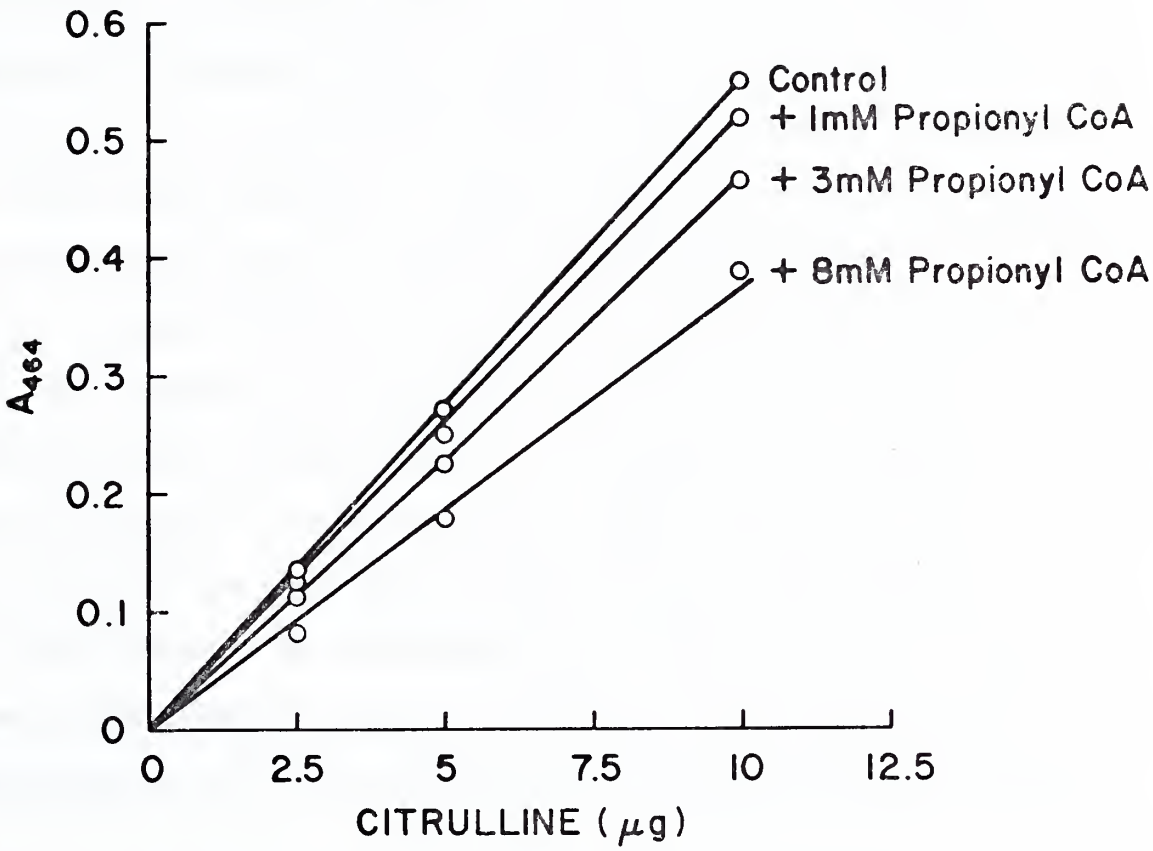

Figure 5. Suppression of the color development reaction used to determine the rate of citrulline synthesis by propionyl CoA. Calculations of CPS I inhibition by propionyl CoA were complicated by the fact that CoA esters inhibit the colorimetric reaction used to measure CPS I activity as well as the enzyme. To obtain an estimate of the amount of color suppression, various concentrations of propionyl CoA were added to known amounts of citrulline, run through the color development process, and then compared to citrulline standards. For each concentration of propionyl CoA, the fractional residual color (i.e. 1 - fractional color suppression) was equal for all concentrations of citrulline: for 1 mM propionyl CoA, 0.95; 3 mM, 0.85, and 8 mM, 0.65.

Results

Selective Inhibition of CPS I Activity in Rat Liver by CoA Esters

The effects of various metabolites on the *in vitro* activities of three urea cycle enzymes were tested. The results shown in Table 1 demonstrate that neither propionate, methylmalonate, tiglate, methylcitrate, nor isoleucine at concentrations of 10 mM inhibited CPS I or OTC activities in rat liver homogenates. Further, neither CoA nor the CoA esters of several of these organic acids affected the activity of OTC or arginase. In sharp contrast to these latter results, however, CPS I activity was regularly and significantly impaired when 10 mM concentrations of CoA, tiglyl CoA, methylmalonyl CoA, or propionyl CoA were added at the point of initiation of the enzyme assay. Table 1 shows data from a single representative experiment in which free CoA reduced activity by 35%, the acyl CoA's by considerably more. Propionyl CoA was regularly the most potent inhibitor, reducing activity by 72% in this study and by an average of 71 ± 5% in experiments with three different rat livers.

Concentration-Dependent and Time-Dependent Inhibition of CPS I Activity

Using propionyl CoA as the model inhibitor in further experiments, I demonstrated that the inhibition of CPS I activity in crude homogenates, in isolated mitochondria, and of purified enzyme is a function of the propionyl CoA concentration. When varying concentrations of propionyl CoA (0-10 mM) were present only during the ten minutes of enzyme assay, inhibition of CPS I activity increased from 35% at 1 mM to 70% at 10 mM (Figure 6). At each concentration of propionyl CoA tested, inhibition

Table 1: Inhibition of urea cycle enzymes by various metabolites in the pathway of isoleucine degradation

	Inhibition of Enzyme Activity (%)		
Compound	CPS I	OTC	Arginase
propionyl CoA	72 (4)	0 (2)	0 (2)
methylmalonyl CoA	66 (2)	0 (2)	*
tiglyl CoA	49 (2)	0 (2)	*
CoA	35 (2)	0 (2)	0 (1)
methylcitrate	4 (2)	0 (2)	*
isoleucine	2 (2)	0 (2)	*
tiglic acid	0 (2)	0 (2)	*
methylmalonic acid	0 (2)	0 (2)	*
propionic acid	0 (2)	0 (2)	0 (1)

Urea cycle enzyme activities in rat liver homogenates were assayed in the presence of 10 mM inhibitor added just prior to the initiation of the assay. The data presented are the averages of duplicate tubes run in a single representative experiment. Control activities were: CPS I 2.6 umoles/hr/mg; OTC 67 umoles/hr/mg; and arginase 500 umoles/hr/mg. The numbers in the parentheses refer to the number of experiments done with each inhibitor at this concentration. * = not tested.

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was slightly greater with the crude homogenates and isolated mitochondria than with purified CPS I, but the difference among the various tissue preparations was small compared to the overall magnitude of the inhibitory effect.

When homogenates were incubated with propionyl CoA prior to initiating the CPS I assay, the inhibitory effect was noted to be time-dependent.

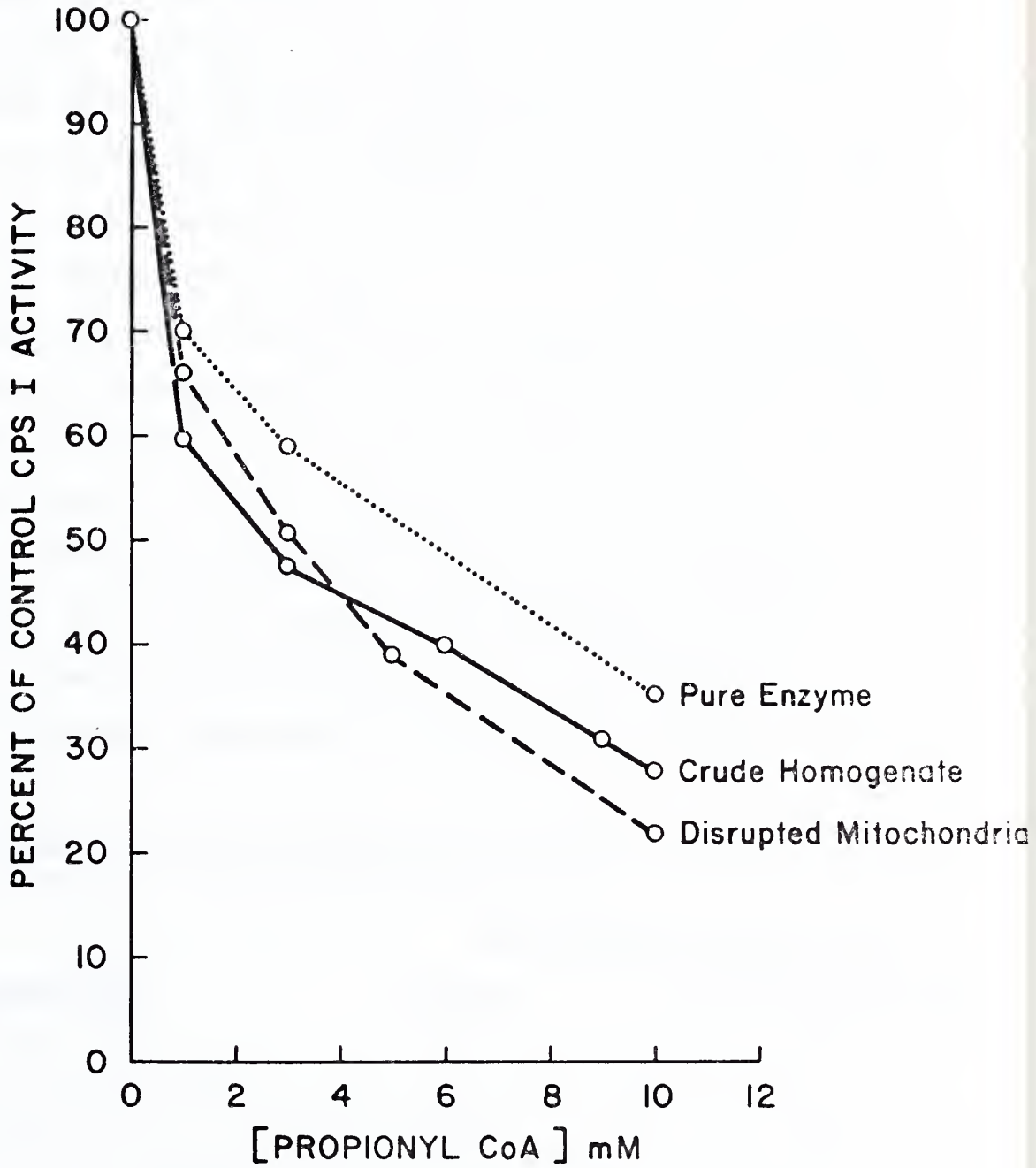

Figure 6. Concentration-dependent inhibition of CPS I by propionyl CoA. CPS I activity in crude homogenates, in disrupted mitochondria, and of pure enzyme from rat liver was measured in the presence of varying concentrations of propionyl CoA (0-10 mM), added at the point of initiation of the enzyme assay. Control activities were: homogenate, 2.4 umoles/hr/mg; mitochondria, 11 umoles/hr/mg; and pure enzyme, 120 umoles/hr/mg.

In the experiment shown in Figure 7, homogenates were incubated with 2.5 mM propionyl CoA and 25 mM NAG for 0-30 minutes and then were assayed for CPS I activity in the presence of 1 mM propionyl CoA. The data demonstrate that the inhibitory effect increased linearly from 45% with no prior incubation (i.e. 10 minutes of total exposure) to 66% when the enzyme was incubated with the inhibitor for 30 minutes prior to assay (i.e. 40 minutes of total exposure. The linearity of the curve suggests that with total exposure times greater than 40 minutes, CPS I activity would likely have been inhibited even more.

A recent paper (94) reported that the inhibition of CPS I activity in isolated rat liver mitochondria by 10 mM propionyl CoA was only about 15% at 30°C, significantly less than the 70% inhibition seen in the present experiments conducted at 37°C. To determine if these disparate results reflected the different temperatures used, I carried out experiments at both 30°C and 37°C. Table 2 shows that CPS I activity in isolated mitochondria was inhibited comparably by 3 mM concentrations of propionyl CoA at both temperatures (43% at 30°C; 40% at 37°C).

Table 2: Effect of incubation temperature on inhibition of CPS I activity in disrupted mitochondria by propionyl CoA

	CPS I Activity (umoles/hr/mg)	
Temperature (°C)	control	+ 3 mM propionyl CoA
30	7.2	4.1
37	4.0	2.4

CPS I activity was assayed in disrupted mitochondria at 30° C and 37° C in the presence and absence of 3 mM propionyl CoA. Data shown are averages of duplicate tubes from one of two identical experiments.

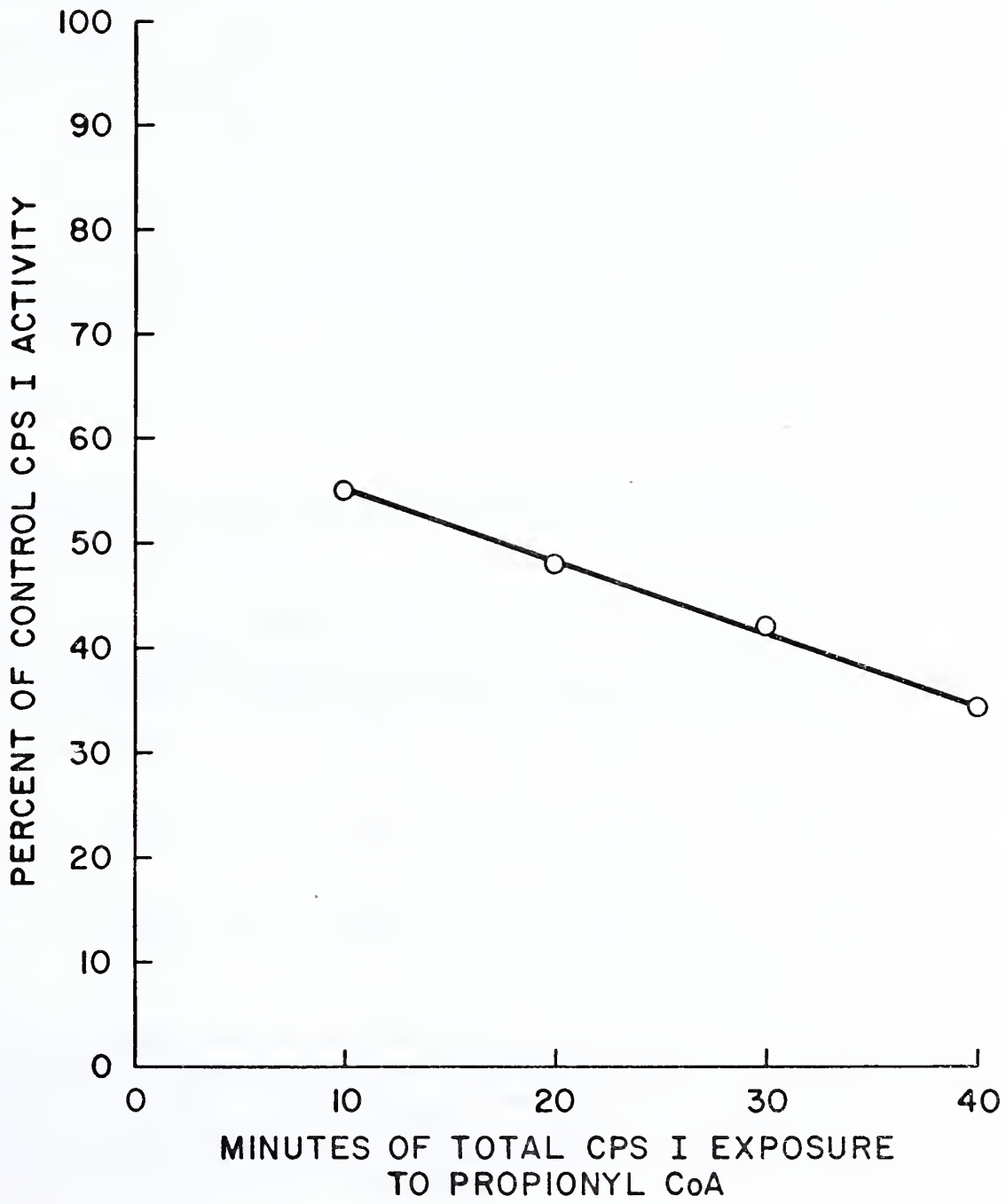

Figure 7. Time-dependent inhibition of CPS I by propionyl CoA. CPS I activity in crude rat liver homogenates was assayed in the presence of 1 mM propionyl CoA after incubation of the enzyme with 2.5 mM propionyl CoA. Control activity was 2.5 umoles/hr/mg and did not vary in control incubations from 0-30 minutes without propionyl CoA.

Mechanism of Propionyl CoA Inhibition

To characterize further the mechanism by which propionyl CoA impairs CPS I activity, Michaelis-Menten kinetic analyses were carried out in rat liver homogenates with each of the enzyme's substrates (ATP, ammonium, and bicarbonate). Double reciprocal transformation of substrate vs. velocity curves yielded linear plots from which the apparent K_m's and V_{max}'s could be calculated. As shown in Table 3, 3 mM propionyl CoA reduced the maximal velocity of CPS I activity by about 50% regardless of which of the enzyme's substrates was varied. Significnatly the apparent K_m's for each substrate were not affected by the propionyl CoA.

Table 3: Kinetic parameters of rat liver CPS I in the presence and absence of 3 mM propionyl CoA

Substrate	V_{max} (umoles/hr/mg)*		K_m (mM)*	
	control	+ 3mM propionyl CoA	control	+ 3mM propionyl CoA
ATP	2.09	1.04	3.2	3.3
NH_4^+	1.95	1.02	0.53	0.45
HCO_3^-	2.53	1.38	3.2	2.7

*Kinetic parameters were determined in rat liver homogenates by varying the concentration of one substrate while keeping the other two substrate concentrations constant. K_m's and V_{max}'s were derived from plots of 1/velocity vs. 1/substrate concentration. Substrate concentrations were varied as follows: for ATP 1, 2, 4, and 20 mM; for NH_4^+ 0.25, 1, 2, 5, and 50 mM; and for HCO_3^- 1, 2, 10, and 50 mM.

To insure that the effect of propionyl CoA on CPS I was not secondary to the described inhibition of NAG synthetase, the NAG concentration in all of our assays was 10 mM, 100 times its reported K_a (98). Furthermore, increasing the concentration of NAG to 100 mM did not decrease the magnitude of the inhibitory effect of propionyl CoA seen in homogenates, isolated mitochondria, or purified enzyme.

In experiments with purified enzyme from rat liver, the addition of dithiothreitol (DTT) to the enzyme after passage through the Sephadex column reduced the degree of inhibition produced by propionyl CoA. Table 4 demonstrates that addition of 2 mM DTT reduced inhibition of CPS I by 3 mM propionyl CoA from 57% to 24%. Increasing the concentration of DTT to 4 mM had no further effect.

To determine the reversibility of propionyl CoA inhibition of CPS I, purified enzyme was incubated with 3 mM propionyl CoA for 15 and 30 minutes, passed through a Sephadex column to remove the propionyl CoA and then assayed. The data shown in Table 5 demonstrate that removal of the propionyl CoA from the assay mixture led to complete loss of its inhibitory effect.

Table 4: Inhibitory effect of 3 mM propionyl CoA with varying concentrations of dithiothreitol (DTT)

	CPS I activity (umoles/hr/mg)	
DTT (mM)	control	+ 3 mM propionyl CoA
0	72	31
2	83	63
4	76	59

CPS I activity was assayed in pure enzyme preparations to which varying concentrations of DTT were added after passage of the enzyme through the Sephadex column used to desalt the enzyme from the storage buffer. Activity with each preparation was measured in the presence and absence of 3 mM propionyl CoA. Data are from one of two identical experiments with similar results.

Table 5: Reversibility of propionyl CoA inhibition of CPS I

	CPS I activity (umoles/hr/mg)	
Incubation time (mins)	control	+ 3 mM propionyl CoA
0	74	--
15	66	70
30	80	76

CPS I activity was measured using pure enzyme preparations incubated for varying times with 3 mM propionyl CoA, passed through a Sephadex column to remove the propionyl CoA and then assayed. Control preparations incubated without propionyl CoA were also assayed.

Results with Human Liver

Finally, very similar results were obtained when CPS I activity was assayed in homogenates of post mortem human liver. Thus, 10 mM propionate had no effect on human hepatic CPS I activity, whereas propionyl CoA, at concentrations of 1 mM or greater, impaired CPS I activity in human liver to an extent virtually identical with that noted for rat liver. For example, exposure of the human liver homogenate to 3 mM propionyl CoA reduced CPS I activity by 48%, an effect indistinguishable from the 52% inhibition observed under identical conditions with rat liver.

Discussion

Hyperammonemia has been a common finding in infants with three disorders of organic acid metabolism, namely propionic, methylmalonic, and α-methylacetoacetic acidemias. The cause of this hyperammonemia is as yet unidentified, but it is likely that its occurrence contributes to the metabolic encephalopathy which is characteristic of children with these disorders. Previous work done in an attempt to link the primary defects in organic acid metabolism with this secondary biochemical abnormality indicates that ureagenesis is impaired either by decreasing the availability or impairing the action of one of the substrates, ATP, or the allosteric activator, NAG, of the CPS I reaction.

The data presented in this paper demonstrate a different kind of inhibitory effect, namely a direct inhibition of CPS I activity by various acyl CoA's. While the organic acids tiglate, propionate, and methylmalonate had no effect on the three urea cycle enzymes measured, their CoA esters significnatly inhibited CPS I activity, but not that of OTC or arginase. In studies using the most potent of these CoA esters, propionyl CoA, inhibition in rat liver homogenates, disrupted mitochondria, and purified enzyme was shown to be concentration-dependent. Inhibition in these preparations increased from about 35% at 1 mM propionyl CoA to 70% at 10 mM.

These results differ from those rep rted in a previous study (92) in which 10 mM propionyl CoA inhibited CPS I activity in disrupted mitochondria by only 15% at 30 C. This difference was shown not to be temperature dependent since in the present experiments significant and equivalent inhibition of CPS I was demonstrated at both 30°C and 37 C.

The difference may be explained if the assays in the previous work were run in the presence of mercaptoethanol or another sulfhydryl reagent since I have shown that these agents protect CPS I from inhibition by propionyl CoA. Furthermore, the control activity measured by these authors was only 50% of that seen at 30°C in the present experiments. This may reflect the presence of another inhibitor in the assays which complicated evaluation of CoA ester inhibition.

The degree of inhibition that was demonstrated with 10 mM propionyl CoA is large enough to account for the hyperammonemia observed in vivo since patients with ammonia intoxication due to primary CPS I deficiencies have been reported with as much as 40% residual CPS I activity (104). In addition, the preliminary studies presented here, in which propionyl CoA was incubated with homogenates prior to assaying CPS I activity, indicate that the inhibition of CPS I by propionyl CoA may also be time-dependent. Thus, the concentration of propionyl CoA needed to achieve marked inhibition of CPS I activity might be less than the 10 mM concentrations regularly employed to achieve 60% inhibition. In fact, incubation of homogenates with 2.5 mM propionyl CoA for 30 minutes followed by assay in the presence of 1 mM propionyl CoA resulted in 66% inhibition of CPS I activity. If the time that propionyl CoA builds up in these disorders, and hence the time of exposure to CPS I, is taken into account, propionyl CoA concentrations closer to the serum concentrations of propionate observed in patients with propionic acidemia will result in 60% inhibition or more.

It should be stressed that my results cannot be explained by the previous data which attributed the hyperammonemia to decreased availability or action of NAG, since the present assays were run in the presence

of 10 mM NAG, 100 times the reported K_a (97). In addition, increasing the concentration of NAG to 100 mM had no effect on the degree of inhibition.

The data obtained in these in vitro studies is consistent with what little in vivo information does exist. First, despite the lack of any measurements of intramitochondrial CoA concentrations in the tissues of patients with these inherited organic acidemias, Stewart and Walser (92) have shown that acyl CoA's (presumably propionyl and methylmalonyl CoA's) increase in mitochondria isolated from rats injected with 20 mmoles/kg propionate or methylmalonate. Since millimolar concentrations of the free acids have been reported in the blood (34), it is plausible that the concentration of the CoA esters employed in our experiments may accumulate intramitochondrially in vivo. Second, and of particular relevance, significantly reduced CPS I activities have been reported in children with these organic acidemias. In 1969, Kirkmann and Keisel (105) described a child with hyperammonemia and a selective deficiency of CPS I activity to 50% of control values who was subsequently shown to have methylmalonic acidemia. More recently, Harris and colleagues reported selective CPS I deficiency to 20% of control values in a child with propionic acidemia (106). It is unlikely that these low CPS I values represent coexisting primary metabolic defects; rather it is likely that they are secondary to the defect in organic acid metabolism. Thirdly, other workers (107,108) have proposed that the hypoglycemia and hyperglycinemia often observed in patients with these organic acidemias may result from the intramitochondrial effects of CoA esters on gluconeogenesis and glycine cleavage, respectively. It is, therefore, attractive to propose that all of the major secondary biochemical abnormalities observed in the organic acidemias may reflect CoA ester-

mediated disturbances of selected mitochondrial functions. Finally, the present data, unlike those of other groups, can explain the hyperammonemia observed in all three disorders since tiglyl, methylmalonyl, and propionyl CoA's all exhibited significant inhibition of CPS I. Inhibition of NAG synthetase, on the other hand, cannot explain the hyperammonemia seen in patients with methylmalonic acidemia since methylmalonyl CoA inhibits this enzyme only to a small degree.

These results provide little information as to the precise biochemical mechanism by which CoA and its derivatives impair hepatic CPS I activity. Results of the Michaelis-Menten analyses indicate that these CoA's do not compete with any of this complex enzyme's substrates for their binding sites since only the V_{max}'s and not the K_m's were affected by propionyl CoA. The ability of dithiothreitol to decrease the degree of inhibition by propionyl CoA suggests that the CoA's act by interacting with sulfhydryl groups on the enzyme, either to alter the active site or change the conformation of the enzyme. The importance of sulfhydryl groups in CPS I has been studied and seems to be related to the stabilization of various conformations. Some sulfhydryl reagents like cysteine result in quick inactivation of the enzyme, which some authors (109) suggest may occur by the breaking of disulfide bonds leading to the dissociation of the dimer. This inactivation requires the presence of NAG (110). Since NAG effects a conformational change on CPS I, this change in tertiary structure may unmask a key sulfhydryl group with which cysteine can react. Other sulfhydryl reagents, such as mercaptoethanol, dithiothreitol, and dithioerythritol, have been shown to stabilize the enzyme (97,111). The difference between these two groups of sulf-

hydryl agents may be due to steric factors or the strength of the -SH groups themselves. In these experiments, dithiothreitol partially protected CPS I from inhibition by propionyl CoA. Propionyl CoA, then, may be acting to inhibit CPS I by forming a propionyl CoA-enzyme complex involving sulfhydryl groups or by accelerating the conformational change to its inactive form. If the latter mechanism is correct, one would predict that preincubation of CPS I with propionyl CoA would lead to inhibition even if the propionyl CoA were to be removed prior to assaying. The inability to demonstrate such an effectin these experiments (Table 5) may be secondary to the absence of NAG in the preincubation mixture, but it may argue against the formation of stable enzyme-inhibitor complexes.

The hyperammonemia seen in patients with inherited organic acidemias appears to occur as the result of the buildup of acyl CoA intermediates in response to an amino acid load. The present data taken together with previous work suggests that the acyl CoA's may work at two sites: namely 1) to inhibit directly CPS I and 2) to decrease NAG availability and secondarily decrease CPS I activity. In addition, factors such as the availability of ATP, increased nitrogen turnover, and the rate of removal of these acyl CoA's may be important in the pathogenesis of the hyperammonemia.

The relationship between the pathogenesis of the hyperammonemia seen in inherietd disorders of organic acid metabolism and that seen in other disorders such as Reye's Syndrome and hypoglycin toxicity (Jamaican vomiting sickness) remains to be demonstrated. Certainly many clinical and biochemical similarities exist including vomiting, lethargy, and coma as well as hypoglycemia and hyperammonemia. If the toxic agent

in each of these disorders inhibits one of the enzymes important for branched-chain amino acid metabolism, significant concentrations of acyl CoA's may accumulate. Alternatively, the toxic agents themselves may be metabolized to short chain fatty acids which could be converted to CoA esters. In either case, this buildup of acyl CoA's may inhibit CPS I and lead to hyperammonemia.

Treatment of children with propionic, methylmalonic, and ∝-methyl-acetoacetic acidemias consists of a diet low in the amino acids that enter the Krebs Cycle through methylmalonate: valine, isoleucine, methionine, and threonine. In patients who are recognized early, institution of such a diet has achieved excellent results (35). In patients where these disorders are unexpected and in whom hyperammonemia occurs, early institution of peritoneal dialysis is essential. If a decrease in NAG availability is important in the pathogenesis of the hyperammonemia, infusion of L-arginine and carbamylglutamate (since NAG has been shown to break down when administered intraperitoneally in rats) may ameliorate the hyperammonemia. To the extent that a direct effect on CPS I by increased levels of acyl CoA's is important *in vivo*, administration of carbamylglutamate is unlikely to decrease ammonia levels, in light of the present work which fails to demonstrate a decrease in inhibition with increased concentrations of NAG *in vitro*. General support in the form of dialysis and removal of the offending amino acids may give some benefit.

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